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Review

Protein phosphorylation and its role in the regulation of Annexin A2 function

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ABSTRACT

Background: Annexin A2 (AnxA2) is a multifunctional protein involved in endocytosis, exocytosis, membrane domain organisation, actin remodelling, signal transduction, protein assembly, transcription and mRNA transport, as well as DNA replication and repair.
Scope of review: The current knowledge of the role of phosphorylation in the functional regulation of AnxA2 is reviewed. To provide a more comprehensive treatment of this topic, we also address in depth the phosphorylation process in general and discuss its possible conformational effects. Furthermore, we discuss the apparent limitations of the methods used to investigate phosphoproteins, as exemplified by the study of AnxA2.
Major conclusions: AnxA2 is subjected to complex regulation by post-translational modifications affecting its cellular functions, with Ser11, Ser25 and Tyr23 representing important phosphorylation sites. Thus, Ser phosphorylation of AnxA2 is involved in the recruitment and docking of secretory granules, the regulation of its association with S100A10, and sequestration of perinuclear, translationally inactive mRNP complexes. By contrast, Tyr phosphorylation of AnxA2 regulates its role in actin dynamics and increases its association with endosomal compartments. Modification of its three main phosphorylation sites is not sufficient to discriminate between its numerous functions. Thus, fine-tuning of AnxA2 function is mediated by the joint action of several post-translational modifications.
General significance: AnxA2 participates in malignant cell transformation, and its overexpression and/or phosphorylation is associated with cancer progression and metastasis. Thus, tight regulation of AnxA2 function is an integral aspect of cellular homeostasis. The presence of AnxA2 in cancer cell-derived exosomes, as well as the potential regulation of exosomal AnxA2 by phosphorylation or other PTMs, are topics of great interest.

1. Introduction to Annexin A2

The annexins are a large protein family with more than 160 unique members identified in a wide range of eukaryotic organisms, including plants, fungi and protists. In mammals, 12 different annexins have been identified, named Annexin A1-A11 and A13 [1,2]. One of the first properties described for annexins was the ability to bind negatively charged phospholipids in the presence of calcium. Subsequently, many additional binding partners and cellular roles have been described, including functions related to membrane traffic and membrane-cytoskeleton dynamics, extracellular receptor activity, signal transduction and RNA binding. Thus, the annexins are now generally considered as multifunctional proteins [1,3–8]. Annexins have also been implicated in numerous pathologies, including cancer, inflammation, cardiovascular diseases and antiphospholipid syndrome [1,7,9–12]. However, it has been difficult to assign a specific pathological role to these proteins.

Annexin A2 (AnxA2), a 39 kDa (36 kDa by SDS-PAGE) member of the annexin family, is expressed in the majority of cells and tissues and binds to numerous ligands. Like the rest of the annexins, AnxA2

Abbreviations: AnxA2, Annexin A2 protein; *anx*A2, *annexin* A2 mRNA; CRM1, chromosome region maintenance-1/exportin-1; CFTR, cystic fibrosis transmembrane conductance regulator protein; EGF, epithelial growth factor; HUVEC, human umbilical vein endothelial cells; LmB, Leptomycin B; MDCK, Madin-Darby canine kidney cells; NES, nuclear export signal; NLS, nuclear localisation signal; PFA, paraformaldehyde; PKA, protein kinase A; PKC, protein kinase C; PM, plasma membrane; PML, promyelocytic leukemia; pSer, phospho-Ser; pSer11AnxA2, Ser11 phosphorylated AnxA2; pSer25AnxA2, Ser25 phosphorylated AnxA2; pThr, phospho-Thr; PTM, post-translational modification; pTyr, phospho-Tyr; pTyr23AnxA2, Tyr23 phosphorylated AnxA2; PC12, rat pheochromocytoma cells; ROS, reactive oxygen species; RTK, receptor Tyr kinase; STAT3/6, Signal transducer and activator of transcription 3/6; SUMO, small ubiquitin-like modifier; tPA, tissue plasminogen activator; TPA, 12-O-tetradecanoylphorbol-13-acetate/phorbol ester; TRPV6, transient receptor potential cation channel subfamily V member 6; UIM, ubiquitin-interacting motif; UTRs, untranslated regions of mRNA; wt, wild type

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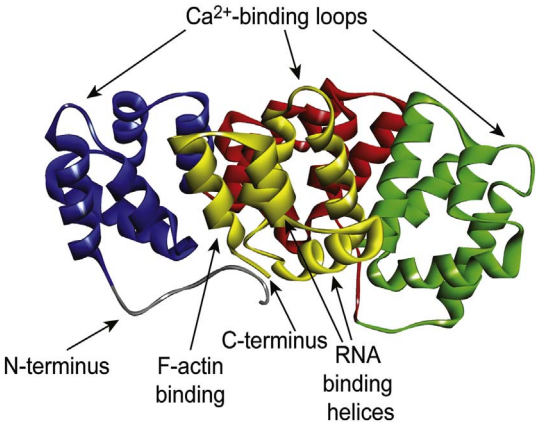

Fig. 1. Annexin structure. Ribbon representation of the crystal structure of bovine AnxA2. The first visible amino acid in this structure is Ser21. PDB code: 4X9P [14]. Domains I, II, III and IV are shown in blue, red, green and yellow, respectively. Calcium-binding loops and the reported RNA- [91] and F-actin- [99] binding sites in AnxA2 are indicated. Note also the close proximity of the N-terminal domain to the C-terminus at the concave side of the protein.

contains the highly conserved "annexin core structure", consisting of four highly homologous domains (named D_I-D_{IV}) of approximately 70 amino acids, which together result in a tightly packed curved structure with a concave and a convex side (Fig. 1). Each domain is made up of 5 short α-helices (named A-E), whereof 4 helices are oriented parallel (A and D, B and E) or anti-parallel (A and B, D and E) to each other, while helix C is oriented nearly perpendicular to the others. The N-terminal domain or "N-terminal tail", which is highly variable between the different members of the annexin family, is located at the concave side of the core structure [1–3,13–15]. However, in the crystal structure of monomeric full-length AnxA2, the electron density of the first 20 amino acids is too low to allow the determination of their structural arrangement. Thus, in the absence of ligand binding, Pro21 is the first visible amino acid in the crystal structure of AnxA2, indicating high flexibility of the N-terminus [13,14].

Like most members of the annexin family, AnxA2 displays the classical property of calcium-dependent lipid binding at its convex side and shows a preference for negatively charged phospholipids. AnxA2 binds several additional ligands including actin, mRNA, tissue plasminogen activator (tPA) and plasminogen [1,3–5,16,17]. Reflecting its multiple functions, AnxA2 is found at various cellular locations. Besides residing soluble in the cytoplasm or associated with the actin cytoskeleton, it is also associated with both the intra- and extracellular sides of the plasma membrane (PM). Additionally, AnxA2 associates with intracellular membrane compartments of both the *endo*- and exocytic pathways [3,18,19]. A minor pool of the protein has also been found in the nucleus [20–23]. Another main ligand for AnxA2 is S100A10 (also called p11), a small EF-hand domain-containing protein. Thus, AnxA2 exists as two main forms: a monomer and a heterotetrameric complex where two molecules of AnxA2 are bound to a S100A10 homodimer [3,24] via a site that comprises the first 12 N-terminal amino acids of AnxA2 [25,26]. AnxA2 is an important regulator of S100A10 as it protects it from ubiquitination and subsequent proteasomal degradation [27]. Conversely, S100A10 exerts a regulatory role in AnxA2 action, increasing its lipid binding affinity and lowering the calcium dependency of its membrane interactions [28]. Thus, the AnxA2-S100A10 complex preferentially associates with membranes, being particularly enriched at the plasma membrane [3]. Accordingly, the functions of the AnxA2-S100A10 complex mainly relates to its membrane localisation, such as its roles as an extracellular receptor, in secretion, or for ion channel function (Section 4.2). The ratio of monomeric and heterotetrameric AnxA2 varies depending on cell type. In Madin-Darby canine kidney cells (MDCK) and porcine intestinal epithelial cells AnxA2 has been reported to be present mainly (90–100%) in the heteroteramer form [29,30], whereas a 1:1 ratio between monomer and tetramer was reported in human fibroblasts [31]. Furthermore, the total amounts of AnxA2 vary between different cells and tissues, with the highest expression levels generally observed in fibroblasts, endothelial and epithelial cells [16]. Thus, the dominant AnxA2 function and localisation are likely to be cell type-dependent.

The multifunctionality of AnxA2 is subjected to complex regulation via ligand binding, subcellular targeting/localisation and post-translational modifications (PTMs). In this review, we will focus on those functional aspects of AnxA2 that are regulated by phosphorylation, the most investigated PTM of AnxA2. With the goal of providing a more comprehensive framework to understand how phosphorylation might regulate AnxA2 functions, we have also included an introduction to phosphorylation in general, as well as a brief discussion of common concerns related to the study of phosphoproteins. Several papers have addressed the possible link between phosphorylation and nuclear localisation of AnxA2. However, due to the lack of consensus between the papers, we have included a more comprehensive discussion of this topic. After reviewing the functional implications of phosphorylation of AnxA2, we will briefly discuss relevant methodological aspects concerning the study of AnxA2 including the use of phospho-mimicking mutants, the effect of GFP-fusion, and the apparent differential exposure of epitopes in different subcellular pools of AnxA2, which may affect antibody detection (Section 8). Other PTMs of AnxA2, as well as possible crosstalk between PTMs of AnxA2 will be addressed briefly.

2. General aspects of protein phosphorylation

PTMs provide a means to regulate protein function, and thus enhance the diversity of the proteome [32]. Phosphorylation is an ubiquitous and extensively studied PTM, which can affect the localisation of a protein, its interaction with other molecules and thus its function. Phosphorylation is involved in the regulation of a multitude of cellular processes, including enzyme activity, metabolism, signal transduction, transcription, cytoskeletal dynamics and cell cycle progression. The complexity of protein phosphorylation is illustrated by the existence of more than 500 protein kinases in the human proteome [33]. Also, at least 70% of mammalian proteins are subjected to phosphorylation, whereof many possess multiple phosphorylation sites [34,35]. Phosphorylation is highly regulated by the activity of kinases, the subcellular localisations of a specific kinase and its substrate, the availability of the phosphorylation site, as well as the presence of consensus sequences or other interaction-stabilising domains. The effects exerted by phosphorylation are also tightly regulated by the activity of the corresponding protein phosphatase. Phosphorylation is most common on Ser, Thr and Tyr residues, and the relative abundances of phospho-Ser (pSer), phospho-Thr (pThr) and phospho-Tyr (pTyr) sites are 86%, 12% and ~2%, respectively [35]. Despite the low abundance of pTyr sites, there is a large interest in this type of phosphorylation as its dysregulation is common in several pathologies, with cancer providing a prime example. In addition, Tyr phosphorylation represents a rapid intracellular response. For example, the pTyr level rapidly increases during the first minute of epithelial growth factor (EGF) stimulation, whereas it may take up to 10 min to reach the maximal levels of pSer/pThr in response to this stimuli [35]. The importance of tight control of Tyr phosphorylation is also reflected by the relatively high numbers of Tyr kinases and phosphatases. It has been estimated that Tyr specific kinases make up ~17% of the kinome (kinase complement of the human genome) [33,36], while ~50% of the human genes encoding phosphatases are Tyr-specific phosphatases [37].

The presence of multiple phosphorylation sites within a protein expands the possibilities and complexities for regulation. For example, the transcription factor nuclear factor of activated T-cells (NFATc2) contains at least 22 phosphorylation sites, which regulate its nuclear localisation, DNA binding, transcriptional activities and *trans*-activation [38]. Phosphorylation at multiple sites within a protein may have